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Molecular Cancer Therapeutics 1

Gefitinib reverses breast cancer resistance protein-mediated drug resistance

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sort of cells is supposed to be one of the determinants of gefitinib sensitivity. Gefitinib inhibits the transporter function of BCRP and reverses BCRP-mediated drug resistance both *in vitro* and *in vivo*. [Mol Cancer Ther 2004;3(9):1119-26]

Abstract

Breast cancer resistance protein (BCRP) is an ATP binding cassette transporter that confers resistance to a series of anticancer agents such as 7-ethyl-10-hydroxycamptothecin (SN-38), topotecan, and mitoxantrone. In this study, we evaluated the possible interaction of gefitinib, a selective epidermal growth factor receptor tyrosine kinase inhibitor, with BCRP. BCRP-transduced human epidermoid carcinoma A431 (A431/BCRP) cells acquired cellular resistance to gefitinib, suggesting that BCRP could be one of the determinants of gefitinib sensitivity in a certain sort of cells. Next, the effect of gefitinib on BCRP-mediated drug resistance was examined. Gefitinib reversed SN-38 resistance in BCRP-transduced human myelogenous leukemia K562 (K562/BCRP) or BCRP-transduced murine lymphocytic leukemia P388 (P388/BCRP) cells but not in these parental cells. In addition, gefitinib sensitized human colon cancer HT-29 cells, which endogenously express BCRP, to SN-38. Gefitinib increased intracellular accumulation of topotecan in K562/BCRP cells and suppressed ATP-dependent transport of estrone 3-sulfate, a substrate of BCRP, in membrane vesicles from K562/BCRP cells. These results suggest that gefitinib may overcome BCRP-mediated drug resistance by inhibiting the pump function of BCRP. Furthermore, P388/BCRP-transplanted mice treated with combination of irinotecan and gefitinib survived significantly longer than those treated with irinotecan alone or gefitinib alone. In conclusion, gefitinib is shown to interact with BCRP. BCRP expression in a certain

Introduction

ATP binding cassette (ABC) transporters, such as P-glycoprotein (1, 2) and MRP1 (3), are involved in multi-drug resistance. They pump out various structurally unrelated anticancer agents in an energy-dependent manner. Breast cancer resistance protein (BCRP, also known as ABCG2) is a half-molecule ABC transporter with an NH₂-terminal ATP binding site and a COOH-terminal transmembrane domain (4-8) and acts as a homodimer (9). BCRP is widely expressed in normal human tissues such as placenta, liver, prostate, small intestine, ovary, colon, and capillary endothelial cells and hematopoietic stem cells (5, 10-12). BCRP is presumed to play a protective role against toxic substrates and metabolites. In addition, overexpression of BCRP confers resistance to anticancer agents such as 7-ethyl-10-hydroxycamptothecin (SN-38), topotecan, 9-aminocamptothecin, and mitoxantrone (7, 13-15). The role of BCRP in clinical drug resistance has not been established, but some reports have shown an association between BCRP expression and poor responses to chemotherapy (16, 17). BCRP has been also shown to be an important determinant of oral bioavailability of BCRP substrate drugs (18).

Gefitinib (ZD1839, Iressa) is an orally active, selective epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor that blocks signal transduction pathways implicated in the proliferation and survival of tumor cells (19, 20). Gefitinib markedly inhibits the autophosphorylation of epidermal growth factor-stimulated EGFR in a broad range of EGFR-expressing human cancer cell lines and xenograft models and has also shown a marked antitumor activity either on its own or in combination in various human tumor xenograft models, especially non-small cell lung cancers (19-22). Gefitinib also showed superior antitumor activity against lung cancers that were refractory to chemotherapy in clinical studies and is now an approved cancer drug in several countries including Japan and the United States (23-27). Further clinical studies of gefitinib in other tumor types, such as breast, head and neck, prostate, breast, gastric, and colorectal tumors, are also ongoing worldwide.

In this study, we evaluated the possible interaction of gefitinib with BCRP. BCRP-transduced A431 cells showed resistance to gefitinib. Gefitinib inhibited the transporter function of BCRP and reversed BCRP-mediated drug resistance both *in vitro* and *in vivo*.

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Materials and Methods

Cells and Cell Culture

A431 human epidermoid carcinoma cells, KB3-1 human epidermoid carcinoma cells, and HT-29 human colon cancer cells were cultured in DMEM supplemented with 10% fetal bovine serum at 37°C in 5% CO₂. K562 human myelogenous leukemia cells and P388 murine lymphocytic leukemia cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum at 37°C in 5% CO₂. A431/BCRP, K562/BCRP, and P388/BCRP cells were established by the transduction of A431, K562, and P388 cells, respectively, with a HaBCRP retrovirus that carries Myc-tagged human BCRP cDNA in the Ha retrovirus vector (9). A431/MDR and K562/MDR cells were established by the transduction of A431 and K562 cells, respectively, with a HaMDR retrovirus that carries human MDR1 cDNA in the same vector (28). The KB/MRP cells were made by introducing the expression vector, pJ30-MRP, containing human MRP1 cDNA into KB3-1 cells (29). The stably transfected cell lines were maintained in drug-free medium for up to 3 months.

Western Blot Analysis

The anti-BCRP polyclonal antibody 3488 was raised by immunizing rabbits with a keyhole limpet hemocyanin-conjugated 20-mer peptide corresponding to amino acid sequence 340 to 359 of human BCRP protein (9). The anti-P-glycoprotein antibody, C219, the anti-MRP1 antibody, MRPm6, and anti- α -tubulin antibody, B-5-1-2, were obtained from Centocor (Malvern, PA), Nichirei (Tokyo, Japan), and Sigma-Aldrich Chemical (St. Louis, MO), respectively. Western blot assays were done as reported previously (9). In brief, cell lysates were solubilized with 2% SDS, 50 mmol/L Tris-HCl (pH 7.5), and 5% 2-mercaptoethanol and resolved by 5% to 20% SDS-PAGE (20 μ g protein per lane). After electrophoresis, proteins were transferred onto nitrocellulose membranes. Blots were incubated with anti-BCRP (8 μ g/mL), anti-P-glycoprotein (2 μ g/mL), anti-MRP1 (2 μ g/mL), or anti- α -tubulin (1 μ g/mL) antibody. After washing, the blots were incubated in 1:500 dilution of the appropriate peroxidase-conjugated secondary antibodies (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom). Membrane-bound peroxidase was visualized using Enhanced Chemiluminescence Plus detection kit (Amersham Pharmacia Biotech). Each blot was exposed for 1 minute (BCRP, P-glycoprotein, and MRP) or 5 seconds (α -tubulin).

Growth Inhibition Assay

Gefitinib was supplied by AstraZeneca UK Ltd. (London, United Kingdom). Cells (2×10^5 – 3×10^5 cells/well) were plated in 12-well dishes and treated with increasing doses of anticancer agents. After 5 days of treatment, cell number was counted with a Coulter counter and drug dose causing 50% inhibition of cell growth (IC₅₀) was determined.

Intracellular Drug Accumulation

The effect of gefitinib on the cellular accumulation of topotecan was determined by flow cytometry. K562 and K562/BCRP cells (5×10^5 cells each) were incubated with 20 μ mol/L topotecan for 30 minutes at 37°C in the absence

or presence of gefitinib (10, 30, and 100 μ mol/L), washed in ice-cold PBS, and subjected to fluorescence analysis using a FACSCalibur (Becton Dickinson, San Jose, CA) with 488 nm excitation.

Intravesicular Transport Assay

Membrane vesicles of K562/BCRP were prepared according to the method described previously (30). The vesicular transport assay was done by a rapid centrifugation technique using ³H-labeled estrone 3-sulfate (E1S), a substrate of BCRP (31). [³H]E1S was purchased from Perkin-Elmer Life Sciences, Inc. (Boston, MA). First, the transport reaction mixture [50 μ L; 50 mmol/L Tris-HCl (pH 7.4), 10 mmol/L MgCl₂, 250 mmol/L sucrose, 10 mmol/L phosphocreatine, 100 μ g/mL creatine phosphokinase, with or without 5 mmol/L ATP, 50 nmol/L [³H]E1S, and membrane vesicles (50 μ g protein)] was kept on ice for 5 minutes and incubated at 37°C for an appropriate time. The reaction was terminated by the addition of ice-cold stop solution [1 mL; 10 mmol/L Tris-HCl (pH 7.4), 100 mmol/L NaCl, 250 mmol/L sucrose]. The membrane vesicles were centrifuged at 18,000 \times g for 10 minutes at 4°C. The pellet was solubilized with 0.1 mol/L NaOH (100 μ L) and neutralized by the addition of 0.1 mol/L HCl. The radioactivity levels were measured by a liquid scintillation counter.

Animal Studies

Six-week-old female CDF1 mice were supplied by Charles River Japan, Inc. (Kanagawa, Japan) and maintained under specific pathogen-free conditions and provided with sterile food and water *ad libitum*. The mice were transplanted with P388 or P388/BCRP cells (10^6 cells per mouse) i.p. from the left flank. For the evaluation of the drug sensitivity of P388/BCRP *in vivo*, irinotecan (30 mg/kg, Yakult Honsha, Tokyo, Japan) and/or gefitinib (150 mg/kg) were given i.p. to the transplanted mice four times with 3-day intervals. The effects of these anticancer agents were evaluated by their effects on survival (the mean survival ratio of treated mice to control mice). Statistical evaluation of a difference between two sets of data was done by a two-tailed Student's *t* test. *P* < 0.05 was considered significant.

Results

Immunoblot Analysis of ABC Transporters

A431/BCRP cells were established by the transduction of A431 cells with the HaBCRP retrovirus and subsequent selection with 51 nmol/L SN-38 for 5 days. K562/BCRP and P388/BCRP cells were also established by a similar SN-38 selection. A431/MDR and K562/MDR were established by the transduction of A431 and K562 cells, respectively, with HaMDR retrovirus and subsequent selection with 4 nmol/L vincristine (VCR) for 7 days. KB/MRP was established by the transfection of KB3-1 cells with pJ30-MRP and subsequent selection with increasing concentrations of doxorubicin. The expressions of BCRP, P-glycoprotein, and MRP1 in these variants were confirmed by Western blot analysis (Fig. 1). BCRP expression

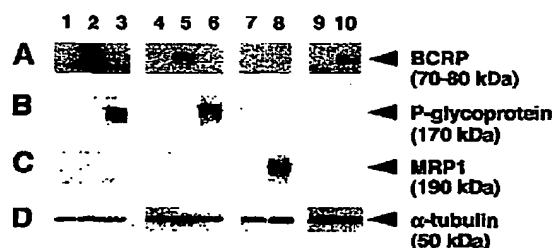


Figure 1. Expression of BCRP, P-glycoprotein, and MRP1 in the transfectants. A, BCRP expression. B, P-glycoprotein expression. C, MRP1 expression. D, α -tubulin expression. Lane 1, A431; lane 2, A431/BCRP; lane 3, A431/MDR; lane 4, K562; lane 5, K562/BCRP; lane 6, K562/MDR; lane 7, KB3-1; lane 8, KB/MDR; lane 9, P388; lane 10, P388/BCRP.

was detected in A431/BCRP, K562/BCRP, and P388/BCRP cells (Fig. 1A). A431/MDR and K562/MDR cells expressed P-glycoprotein (Fig. 1B) and MRP1 expression was detected in KB/MDR cells (Fig. 1C).

Drug Resistance of ABC Transporter-Expressing Cells

The sensitivity of ABC transporter-expressing cells to anticancer agents was examined using a growth inhibition assay (Fig. 2; Table 1). A431/BCRP and K562/BCRP cells acquired resistance to SN-38, and A431/MDR and K562/MDR cells acquired resistance to VCR as compared with respective parental cells (Fig. 2A and C; Table 1). P388/BCRP cells acquired resistance to SN-38 (Table 1). KB/MDR cells also acquired ~10-fold higher resistance to etoposide (VP-16) than the parental cells (Table 1). As shown in Fig. 2B, A431/BCRP cells showed cross-resistance to gefitinib. The IC_{50} value of gefitinib in A431/BCRP cells was 75.8 nmol/L, which was 9.07-fold higher than that in the parental A431 cells (8.43 nmol/L; Fig. 2B; Table 1). A431/MDR cells also showed a 2.36-fold higher resistance to gefitinib than A431 cells (Table 1). These results suggest that BCRP and P-glycoprotein are involved in the cellular resistance to gefitinib in A431 cells. In contrast, K562/BCRP, K562/MDR, and P388/BCRP cells did not show gefitinib resistance (Fig. 2D; Table 1).

Reversal of BCRP-Mediated Drug Resistance by Gefitinib

The effects of gefitinib on BCRP-mediated drug resistance were examined. As shown in Fig. 3A, ≤ 1 μ mol/L gefitinib strongly enhanced the cytotoxicity of SN-38 in K562/BCRP cells but not in K562 cells. Gefitinib alone at 1 μ mol/L showed little growth inhibitory effects on K562 or K562/BCRP cells (Fig. 2D). Gefitinib also potentiated the cytotoxicity of SN-38 in P388/BCRP cells (Fig. 3B). Next, effects of gefitinib on drug resistance in either P-glycoprotein-expressing or MRP1-expressing cells were examined to evaluate transporter specificity. Gefitinib enhanced VCR cytotoxicity in K562/MDR cells in a dose-dependent manner but showed no effect on MRP1-mediated etoposide (VP-16) resistance in KB/MDR cells (Fig. 3C and D). Possible effect of gefitinib on cells that endogenously express

BCRP was examined using human colon cancer cells HT-29 (Fig. 4). As shown in Fig. 4B, gefitinib at 0.3 and 1 μ mol/L increased the SN-38 cytotoxicity of HT-29 cells. Gefitinib alone showed no effect on the growth of HT-29 cells at 1 μ mol/L (data not shown).

Effect of Gefitinib on BCRP-Mediated Transport

The effects of gefitinib on the intracellular accumulation of topotecan in BCRP-expressing cells were examined by flow cytometric analysis. After incubating cells with 20 μ mol/L topotecan, significant increases in fluorescence intensity occurred in K562 cells (Fig. 5A). However, the cellular fluorescence of K562/BCRP cells increased only marginally, suggesting that BCRP exports topotecan out of the cells (Fig. 5B). Gefitinib treatment enhanced the intracellular accumulation of topotecan in K562/BCRP cells in a dose-dependent manner (Fig. 5D, F, and H) but showed no effect on that in parental K562 cells (Fig. 5C, E, and G). Similar effects of gefitinib were observed in P388/BCRP cells (data not shown). Next, the effects of gefitinib on the BCRP-mediated transport of E1S were examined using membrane vesicles from K562/BCRP cells. As shown in Fig. 6, ATP-dependent uptake of [3 H]E1S was inhibited by gefitinib in a dose-dependent manner. The IC_{50} value of gefitinib to inhibit the pump function of BCRP was 1.01 ± 0.30 μ mol/L. No ATP-dependent E1S uptake was observed in K562 membrane vesicles (data not shown). Taken together, these results suggest that gefitinib inhibits BCRP-mediated transport.

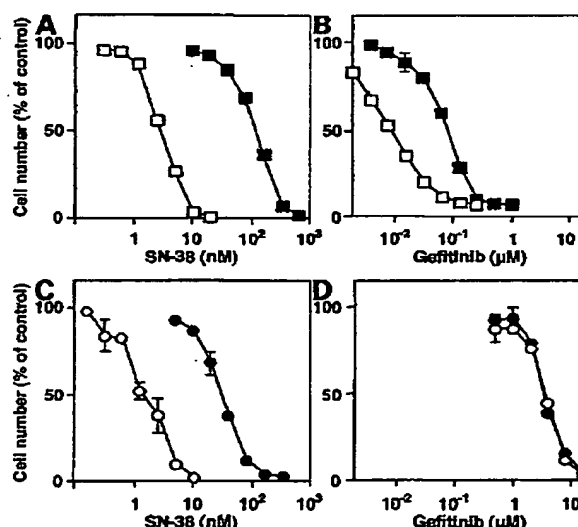


Figure 2. A, SN-38 sensitivity of A431 and A431/BCRP cells. B, gefitinib sensitivity of A431 and A431/BCRP cells. C, SN-38 sensitivity of K562 and K562/BCRP cells. D, gefitinib sensitivity of K562 and K562/BCRP cells. Cells were cultured for 5 days with increasing concentrations of SN-38 (A and C) or gefitinib (B and D). Cell numbers were determined with a Coulter counter. Points, mean of triplicate determinations; bars, SD. \square , A431; \blacksquare , A431/BCRP; \circ , K562; \bullet , K562/BCRP.

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Table 1. Drug resistance of ABC transporter-expressing cells

Cell	IC ₅₀ (nmol/L)				Degree of Resistance			
	SN-38	VCR	Gefitinib	VP-16	SN-38	VCR	Gefitinib	VP-16
A431	2.91 ± 0.08	0.15 ± 0.02	8.43 ± 1.15					
A431/BCRP	120 ± 1.15	ND	75.8 ± 5.57		41.4	—	9.07	
A431/MDR	ND	57.6 ± 1.83	19.7 ± 0.38		—	380	2.36	
K562	1.68 ± 0.56	0.07 ± 0.005	3,480 ± 52.9					
K562/BCRP	30.6 ± 0.82	ND	3,250 ± 108		18.2	—	0.93	
K562/MDR	ND	23.8 ± 3.65	4,140 ± 137		—	367	1.19	
P388	15.2 ± 1.71	ND	2,420 ± 191					
P388/BCRP	194 ± 9.07	ND	4,130 ± 214		12.8	—	1.71	
KB3-1				111 ± 2.67				
KB/MRP				1,200 ± 151				10.8

NOTE: Cells were cultured for 5 days in the absence or presence of increasing concentrations of the indicated anticancer agents. Cell numbers were determined with a Coulter counter and IC₅₀ values were calculated. The degree of resistance is the ratio of the IC₅₀ values for BCRP-transduced, MDR1-transduced, or MRP-transduced cells divided by those for the parental cells. Data are means ± SD from triplicate determinations. ND, not determined; VP-16, etoposide.

Reversal of BCRP-Mediated Drug Resistance by Gefitinib *In vivo*

To evaluate the circumvention of drug resistance by gefitinib *in vivo*, mice (six per group) were transplanted i.p. with either P388 or P388/BCRP cells (10⁶ cells per mouse)

and subsequently treated with irinotecan (30 mg/kg) and/or gefitinib (150 mg/kg) four times on days 1, 4, 7, and 10 successively. The survival time of P388-transplanted mice without treatment was 9.3 ± 0.5 days. The survival time of the P388-inoculated mice, treated with irinotecan alone, was 26.7 ± 2.9 days, with a treated versus control of 287%. Gefitinib was not effective against P388 cells *in vivo* (Table 2). Treatment of neither irinotecan alone nor gefitinib alone was effective in P388/BCRP-transplanted mice. These results suggest that BCRP is responsible for irinotecan resistance *in vivo*. However, coadministration of irinotecan with gefitinib extended the survival of P388/BCRP-inoculated mice, with a treated versus control of 140% (*P* = 0.017; Table 2). These results indicate that gefitinib reverses BCRP-mediated irinotecan resistance *in vivo*.

Discussion

In this study, we describe the interaction of gefitinib with BCRP. A431/BCRP cells showed higher resistance to gefitinib than the parental cells (Fig. 2B; Table 1), suggesting

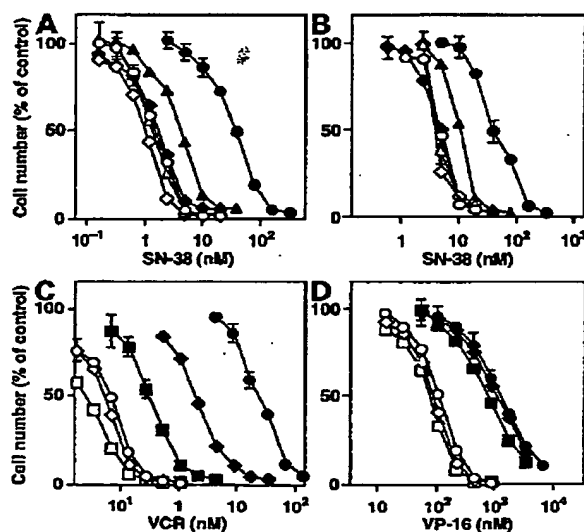


Figure 3. Effects of gefitinib on ABC transporter-mediated drug resistance. Cells were cultured for 5 days with increasing concentrations of anticancer agents in the absence or presence of gefitinib. Cell numbers were determined with a Coulter counter. Points, mean of triplicate determinations; bars, SD. A, effect of gefitinib on the SN-38 sensitivity of K562 (O, Δ, and ◇) and K562/BCRP (●, ▲, and ◆) cells. B, effect of gefitinib on the SN-38 sensitivity of P388 (O, Δ, and ◇) and P388/BCRP (●, ▲, and ◆) cells. C, effect of gefitinib on the VCR sensitivity of K562 (O, Δ, and ◇) and K562/MDR (●, ▲, and ◆) cells. D, effect of gefitinib on the etoposide (VP-16) sensitivity of KB3-1 (O, Δ, and ◇) and KB/MRP (●, ▲, and ◆) cells. O and ●, without gefitinib; Δ and ▲, 0.3 μmol/L gefitinib; ◇ and ◆, 1 μmol/L gefitinib; □ and ■, 3 μmol/L gefitinib.

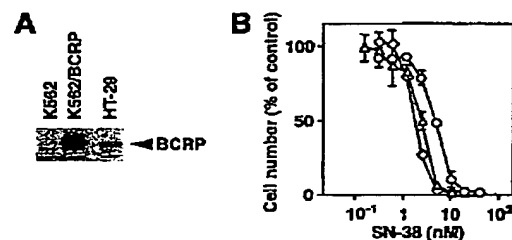


Figure 4. Effect of gefitinib on cells that express endogenous BCRP. HT-29 cells were cultured for 5 days with increasing concentrations of SN-38 in the absence or presence of gefitinib. A, BCRP expression. B, effect of gefitinib on the SN-38 sensitivity of HT-29 cells. O, without gefitinib; Δ, 0.3 μmol/L gefitinib; ◇, 1 μmol/L gefitinib.

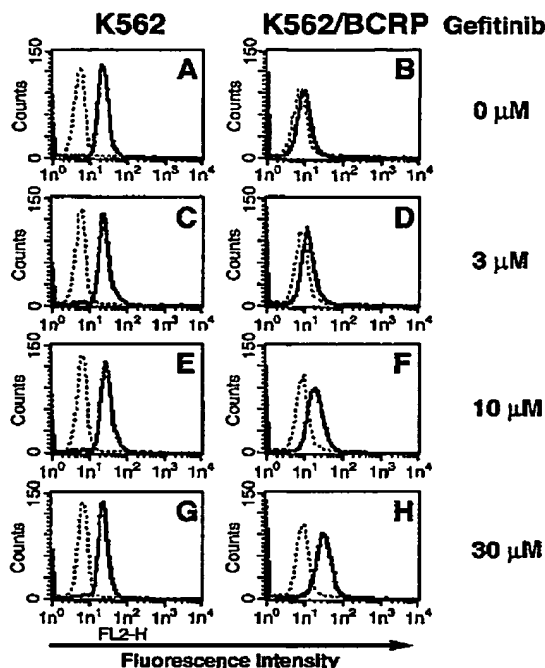


Figure 5. Effect of gefitinib on the intracellular accumulation of topotecan in K562 (A, C, E, and G) and K562/BCRP (B, D, F, and H) cells. Cells were incubated in the absence (dotted lines) or presence (bold lines) of 20 $\mu\text{mol/L}$ topotecan in combination with gefitinib at doses of 0 $\mu\text{mol/L}$ (A and B), 3 $\mu\text{mol/L}$ (C and D), 10 $\mu\text{mol/L}$ (E and F), or 30 $\mu\text{mol/L}$ (G and H). Cellular topotecan content was measured by fluorescence-activated cell sorting.

that BCRP may be one of the determinants of gefitinib resistance in a certain sort of cells. Reversal of BCRP-mediated drug resistance by gefitinib was also observed in both K562/BCRP and P388/BCRP cells (Fig. 3A and B). Gefitinib increased the cellular accumulation of topotecan in K562/BCRP (Fig. 5) and suppressed the ATP-dependent transport of E1S in membrane vesicles from K562/BCRP cells (Fig. 6). These findings suggest that gefitinib inhibits the transporter function of BCRP and increase the cytotoxicity of BCRP substrate anticancer agents.

As described above, A431/BCRP cells acquired gefitinib resistance, whereas K562/BCRP cells did not. We do not know the reason why A431/BCRP is resistant to gefitinib but K562/BCRP is not. K562/BCRP and A431/BCRP cells expressed similar levels of BCRP and showed similar levels of SN-38 resistance. Therefore, difference in the BCRP expression levels is not the reason for this discrepancy. In our preliminary observation, BCRP-transduced human non-small cell lung cancer PC-9 (PC-9/BCRP) cells also acquired gefitinib resistance. PC-9 and A431 are highly sensitive to gefitinib (32). PC-9 and A431 cells express EGFR and their growth is supposed to be EGFR dependent. Gefitinib seems to show growth inhibitory effect against PC-9 and

A431 through, at least in part, EGFR-dependent signaling pathway. In contrast, K562/BCRP and P388/BCRP cells showed similar levels of gefitinib sensitivity to their parental cells (Table 1). K562 and P388 cells do not express EGFR and are relatively resistant to gefitinib. Mechanism of gefitinib cytotoxicity against K562 and P388 is not clear because EGFR-dependent pathway does not function in these cells. Growth inhibitory effects of gefitinib in such EGFR-negative cells should be independent of epidermal growth factor signaling and EGFR phosphorylation; therefore, some other low-affinity molecular targets of gefitinib may exist. Anyway, we could hypothesize from the current study that BCRP expression confers gefitinib resistance in cells that express EGFR and grow EGFR dependently. BCRP could be an important determinant for anticancer activity of gefitinib in clinical situations.

There are several possible mechanisms in the enhancement of SN-38 cytotoxicity in A431/BCRP by gefitinib. One is the competitive inhibition of the BCRP-mediated SN-38 efflux by gefitinib. To clarify this mechanism, direct transport experiments such as membrane vesicle transport assays or drug accumulation/efflux studies are currently ongoing in our laboratory. Whether gefitinib is a member of the BCRP substrate group of anticancer agents is a very important subject. Other tyrosine kinase inhibitors, CI1033 and imatinib mesylate, have been shown to inhibit the function of BCRP (33, 34). The second possible mechanism is the inhibition of BCRP function through the inhibition of protein phosphorylation. At present, no report exists on the role of possible BCRP phosphorylation on the transporter function. In addition, other unknown gefitinib-sensitive protein phosphorylation pathway may exist and regulate the drug efflux function of BCRP. In this study, we have also shown that A431/MDR cells acquired low gefitinib resistance, but K562/MDR cells did not. These results suggest that gefitinib resistance mediated by ABC transporters depends, at least in part, on the EGFR-dependent growth of tumor cells or cellular gefitinib sensitivity. The third

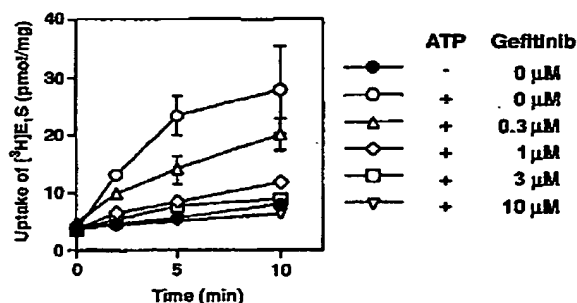


Figure 6. Effect of gefitinib on the intravesicular uptake of E1S. Membrane vesicles from K562/BCRP were incubated at 37°C with 50 nmol/L [^3H]E1S and increasing concentrations of gefitinib in the absence (●) or presence (○, △, ◇, □, and ▽) of 5 mmol/L ATP. Points, mean of triplicate determinations; bars, SD.

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Table 2. Circumvention of BCRP-mediated drug resistance *in vivo* by gefitinib

Tumor	Irinotecan (mg/kg)	Gefitinib (mg/kg)	Survival (d)*	T/C (%) [†]	T/I (%) [‡]
P388	0	0	9.3 ± 0.5	100	
	30	0	26.7 ± 2.9	287 [§]	100
	0	150	9.3 ± 0.8	100	
	30	150	27.8 ± 3.1	299 [§]	104
P388/BCRP	0	0	11.3 ± 1.0	100	
	30	0	11.5 ± 0.5	102	100
	0	150	10.2 ± 1.2	90	
	30	150	15.8 ± 1.8	140 [§]	137

*Data are means ± SD.

[†]The ratio of survival time to that of mice without treatment.[‡]The ratio of survival time to that of irinotecan-treated mice.[§]*P* < 0.01 compared with control.[¶]*P* = 0.017 compared with control.

explanation would be that gefitinib and other kinase inhibitors may inhibit energy production needed for pump function by competing with ATP for binding to BCRP. This possibility will also be examined in the future.

On the other hand, the reversal activity of BCRP-mediated drug resistance by gefitinib in K562/BCRP and P388/BCRP cells suggests that gefitinib can interact with BCRP in these cell types, too. The BCRP inhibitory effect of gefitinib may be also explained by competitive inhibition of BCRP-mediated drug export. We examined the effect of gefitinib on SN-38 resistance of K562/BCRP and P388/BCRP cells at gefitinib concentrations of 0.3 and 1 μmol/L (Fig. 3A and B). Gefitinib at 0.3 and 1 μmol/L showed good reversal activity of the SN-38 resistance of K562/BCRP and P388/BCRP cells (Fig. 3A and B). K562/BCRP and P388/BCRP did not show any SN-38 resistance in the presence of 1 μmol/L gefitinib. Reportedly, the maximum plasma concentrations resulting from clinically relevant gefitinib doses were 0.5 to 1 μmol/L (27). This suggests that the blood levels of gefitinib would be sufficient for the reversal of BCRP-mediated drug resistance *in vivo*. In general, doses of anticancer agents used in mice are much higher than those in human. Kunimoto et al. (35) have examined the antitumor activities of irinotecan in mice at doses of 100 or 200 mg/kg. In this study, irinotecan was given to mice at similar dosage (30 mg/kg/d for 4 days, 120 mg/kg in total). Gefitinib has shown the antitumor activities in xenografts of A549, Dul45, A431, CR10, HCT15, HT-29, Lovo, KB, or HX62 cells at doses of 12.5 to 200 mg/kg/d (36). In this study, gefitinib was given to mice at 150 mg/kg. Gefitinib dosage may be relatively high in our study because inhibitor of transporter (gefitinib) is used generally at higher dosage than the substrate of transporter (irinotecan). Evaluation of clinical significance of coadministration of irinotecan and gefitinib must await further clinical investigations.

Although gefitinib seems to be effective in reversal of BCRP-mediated resistance to irinotecan *in vivo*, the reversal

is not complete (~50% of the control). BCRP transfectants used in this study have been established after the selection of the transfected cells with appropriate anticancer agents for only several days. Therefore, other drug resistance mechanism such as P-glycoprotein or DNA repair pathway would not exist. Reason for the incomplete reversal of the drug resistance *in vivo* may be attributable to the bioavailability and pharmacokinetics/pharmacodynamics of the drugs.

As both irinotecan and gefitinib are effective against non-small cell lung cancer, they may be used in combination in clinical practice. The pharmacologic interaction of these agents, through the inhibition of BCRP, should be considered if such regimens are employed. Previously, interaction between irinotecan and gefitinib was reported in human colorectal cancer cell lines (37). In our *in vivo* study, no weight loss was observed in irinotecan-alone-treated or gefitinib-alone-treated mice. However, combined treatment of irinotecan and gefitinib showed toxicity in mice; especially, P388-transplanted mice lost 4 g on day 15 and regained on day 18. This may be due to the inhibition of endogenous BCRP by gefitinib. Gefitinib may also increase the cytotoxicity of irinotecan in BCRP-expressing normal tissues.

In summary, we have shown that gefitinib interacts with BCRP. BCRP expression is supposed to be one of the determinants of gefitinib sensitivity in a certain sort of cells. Gefitinib inhibits the transporter function of BCRP and reverses BCRP-mediated drug resistance both *in vitro* and *in vivo*.

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